Relationship of salivary and plasma cortisol levels in preterm infants: results of a prospective observational study and systematic review of the literature

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Abstract: BACKGROUND AND OBJECTIVES: (1) To investigate the relationship of salivary and plasma cortisol levels in preterm infants with a focus on the usability of salivary cortisol in diagnostic work-up of infants at risk of adrenal insufficiency. (2) To perform a systematic review addressing this question. METHODS: Clinical study: We conducted a prospective observational single-center study in preterm infants. We analyzed plasma and saliva cortisol concentrations by enzyme immunoassay. Correlation analysis was used to determine the relation between salivary and plasma cortisol levels and the agreement of the measurement methods was analyzed according to Bland-Altman. Systematic review: A systematic literature search (PubMed and Embase) on the relationship of salivary and plasma cortisol levels in neonates was performed in November 2012. RESULTS: Clinical study: We enrolled 58 preterm infants (median (interquartile range) gestational age at birth was 31.4 (28.1-32.7) weeks, birth weight 1,340 (974-1,745) g, respectively). Correlation analyses revealed a relationship of plasma cortisol and salivary cortisol levels. Rank correlation coefficient was 0.6. Estimating plasma cortisol levels based on measured salivary cortisol levels showed poor agreement of the two methods for determining plasma cortisol levels (direct and via salivary cortisol). Sensitivity and specificity of salivary cortisol for the detection of adrenal insufficiency were 0.66 and 0.62, respectively. Systematic review: Six studies in preterm infants and term neonates depicting the correlation of salivary and plasma cortisol were identified with a range of saliva-plasma correlation coefficients from 0.44 to 0.83. CONCLUSIONS: Substitution of plasma cortisol by salivary cortisol determination cannot be recommended in preterm infants because of unsatisfactory agreement between methods.

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Relationship of Salivary and Plasma Cortisol Levels in Preterm Infants: Results of a Prospective Observational Study and Systematic Review of the Literature

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Key Words
Infant, premature · Newborn · Cortisol · Plasma · Saliva · Adrenal insufficiency · Sensitivity and specificity

Abstract
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Introduction

The assessment of hypothalamic-pituitary-adrenal function in very preterm infants is important as immaturity of the hypothalamic-pituitary-adrenal axis can lead to inadequate adrenal function in the face of acute illness [1, 2] and corticosteroid therapy may influence adrenal

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function. For the purpose of cortisol measurement in preterm infants, easily available and workable methods are needed, ideally not inducing pain and stress to this vulnerable population [3]. Saliva collection is easy to perform and reliable in adults and older children wherefore salivary concentrations of cortisol reflecting free cortisol concentrations in plasma is used to evaluate adrenal cortex function [4–7]. The best method of assessing adrenal function in preterm infants still is under debate but salivary cortisol measurement seems to be a promising diagnostic feature for these infants, too [8–10].

We aimed at investigating the relationship of salivary and plasma cortisol levels in preterm infants with special regard to usability of salivary cortisol in diagnostic workup of infants at risk of adrenal insufficiency and at comparing our results to a second cohort of older children and young adults. Additionally, we performed a systematic review of studies addressing the relationship of salivary and plasma cortisol levels in preterm infants and term neonates with a focus on the usefulness of salivary cortisol in a clinical context.

Patients, Materials and Methods

Clinical Study

This prospective observational study was performed at Tuebingen University Children's Hospital. The institutional review board approved the study. All subjects' parents gave written informed consent.

Patients

Preterm infants with a gestational age (GA) at birth <34 weeks or a birth weight (BW) <1,500 g and a postmenstrual age <36 weeks at sampling time were eligible. Exclusion criteria were dysmorphic features or congenital malformations that potentially involve the function of the pituitary or adrenal glands.

For detection of potential age-related differences in the relationship of plasma and salivary cortisol levels, a second cohort of children and young adults was investigated. These children had blood samples taken in the outpatient clinic of our pediatric endocrinology department in September 2011 for clinical diagnostic reasons.

Materials

Saliva and Blood Collection. Saliva samples were collected during regular care rounds in the awake infant using two little cellulose sponges (Visitec Eye Sponge®) [11]. Sampling was carried just before blood withdrawal by venipuncture without any additional stimulation of saliva production. Saliva was extracted from the sponges by immediate centrifugation (15 min at 3,500 *g*).

Salivary Cortisol Assay. Cortisol in saliva was measured by cortisol enzyme-linked immunosorbent assay, ELISA (IBL International GmbH, Hamburg, Germany) developed for determination of free cortisol in human saliva and of total cortisol in diluted serum or plasma. Intra-assay variation (CV) was 5.5% at 0.47 μg/dl. Inter-assay variation was reported to be 8.8% at 0.54 μg/dl by the manufacturer. The analytical sensitivity of the assay is at 0.005 μg/dl. Samples were frozen at –20°C before processing. After thawing and mixing, samples were centrifuged 10 min at 2,000 *g* to remove particulate material. For assaying, 50 μl of the supernatant was used.

Plasma Cortisol Assay. Cortisol in plasma was measured by the identical Cortisol ELISA (IBL International GmbH) as in saliva. Intra-assay variation (CV) was 6.1% at 4.0 μg/dl and 9.2% at 1.7 μg/dl. Inter-assay variation was reported to be 13% at 2.7 μg/dl by the manufacturer. Plasma was diluted with assay buffer to 1:50 before testing.

Statistical Analyses

Linear regression was used to investigate the relationship between measured salivary cortisol levels and plasma cortisol levels. A linear slope fitted best for the adjustment of salivary and plasma cortisol levels. This linear equation was used to convert saliva to plasma levels. Agreement between plasma cortisol levels obtained via conversion of saliva cortisol data and directly measured plasma levels was determined according to Bland and Altman [12].

Correlation between salivary and plasma cortisol levels was analyzed using Spearman's rank correlation coefficient. Additionally, sensitivity and specificity of salivary cortisol for the detection of adrenal insufficiency were calculated. A directly measured plasma cortisol <3.7 μg/dl was defined as a threshold value indicating adrenal hypofunction in preterm infants. This threshold was chosen based on data by Ng et al. [13] investigating reference values for serum cortisol concentration in preterm infants and is consistent with the 10th centile for serum cortisol in this study population. If necessary, normal distribution of data was tested and non-parametric tests were used if the assumption did not apply.

Systematic Review of the Literature

Literature Search

In November 2012, we performed an electronic literature search in PubMed and Embase (from inception to November 2012). The strategy for the search in PubMed was as follows: 'Hydrocortisone' [Mesh] and 'Saliva' [Mesh] and ('humans' [MeSH Terms] and ('infant' [MeSH Terms]). For the Embase search we used the following terms: 'saliva' and 'cortisol' and 'newborn'. No language restrictions were applied. All identified reports were checked for references of additional relevant studies.

Eligibility and Data Extraction

Studies were included if the relationship of salivary and plasma cortisol was investigated in preterm infants or term neonates. Title, abstract and full text of retrieved reports – the latter only if necessary for definite decision of eligibility – were assessed for available data on the relationship of salivary and plasma cortisol in preterm infants and term neonates.

Data were extracted with the use of a standardized form. We extracted the following information: characteristics of the study (e.g. language of publication, year of publication), characteristics of the study population, number of infants investigated, procedures used for saliva collection, number of analyzed paired saliva plasma samples, assays used for salivary cortisol determination, correlation coefficients for the relationship of salivary and plasma cortisol levels.

Table 1. Patient characteristics of preterm infants

	Enrolled preterm infants	Analyzed preterm infants
Number	58	30
Male/female	24/34	12/18
Gestational age at birth, weeks	31.4 (28.1–32.7)	31 (28.1–33.2)
Birth weight, g	1,340 (974–1,745)	1,270 (953–1,894)
Antenatal corticosteroids, n/total n (%)	53/58 (91.4)	28/30 (93.3)
Time on mechanical ventilation via endotracheal tube, days	0 (0–1)	0 (0–1)
Time on nasal CPAP or mechanical ventilation via endotracheal tube, days	5 (1–32)	5.5 (1–32.3)
Postmenstrual age at discontinuation of supplemental oxygen, weeks	32.1 (30.4–33.43)	31.9 (29.8–33.5)
CRIB score	1 (0–1)	1 (0–1)
Bronchopulmonary dysplasia[a], n/total n (%)	3/58 (5.2)	2/30 (6.7)
Necrotizing enterocolitis (any), n/total n (%)	0/58 (0)	0/30 (0)
Intraventricular hemorrhage grade 1–2, n/total n (%)	2/58 (3.5)	1/30 (3.3)
Intraventricular hemorrhage grade 3–4, n/total n (%)	0/58 (0)	0/30 (0)
Retinopathy of prematurity (any), n/total n (%)	1/58 (1.7)	0/30 (0)
Postmenstrual age at sampling time, weeks		33 (30–34.9)
Weight at sampling time, g		1,352 (1,115–2,084)
Nasal CPAP at sampling time, n/total n (%)		10/30 (33.3)

Data are presented as median (IQR) where applicable. [a] Bronchopulmonary dysplasia was defined as receiving supplemental oxygen or positive airway pressure at a postmenstrual age of 36 weeks.

Results

Clinical Study

Patient Characteristics and Cortisol Levels

The study was conducted from March to October 2011. Out of 84 screened preterm infants, 58 were enrolled (see online suppl. fig. 3; for all online suppl. material, see www.karger.com/doi/10.1159/000357555). For patient characteristics see table 1. In 30/58 patients (51.7%), we were able to collect at least 50 μl of saliva (median (interquartile range, IQR) sample volume 53 (10–80) μl) which was the minimal amount of saliva for salivary cortisol determination without further dilution. We compared salivary and plasma cortisol levels in these 30 preterm infants. Plasma cortisol ranged from 2.3 to 15.8 μg/dl (mean ± SD 6.7 ± 4.2 μg/dl) and salivary cortisol from 0.16 to 0.98 μg/dl (mean ± SD 0.43 ± 0.24 μg/dl), respectively.

In the same time period, paired saliva blood samples were investigated from a second cohort of 19 additional subjects aged between 5 and 20 years (median (IQR) age 12.2 (7.9–14.3) years).

Comparison of the Two Methods

Regression analyses revealed that assuming a linear relationship fitted best. The following equations were found to be most suitable for converting salivary cortisol levels into plasma levels: Plasma cortisol calculated from salivary cortisol = (salivary cortisol – 0.19)/0.04) for preterm infants ($R^2 = 0.41$); = (salivary cortisol + 0.04)/0.05) for children ($R^2 = 0.80$).

Agreement between calculated plasma cortisol levels (via conversion from salivary cortisol data) and directly measured plasma cortisol was low for both cohorts (fig. 1).

Sensitivity and specificity of salivary cortisol for the detection of adrenal insufficiency in preterm infants were 0.66 (95% confidence interval (CI) 0.3–0.93) and 0.62 (95% CI 0.38–0.82), respectively.

For comparison of our own data to data found in the systematic literature review, we additionally calculated rank correlation coefficients according to Spearman for non-normally distributed data. R was 0.6 (95% CI 0.31–0.79) for preterm infants and 0.8 (95% CI 0.53–0.92) for children, respectively (fig. 2).

In the preterm infant cohort we observed several subjects in which plasma cortisol levels in the lower range were associated with unexpectedly high salivary cortisol levels (fig. 2a).

Systematic Review of the Literature

The literature search retrieved 221 potentially relevant results (213 in PubMed and an additional 8 results in Embase that were not detected in PubMed) of which six stud-

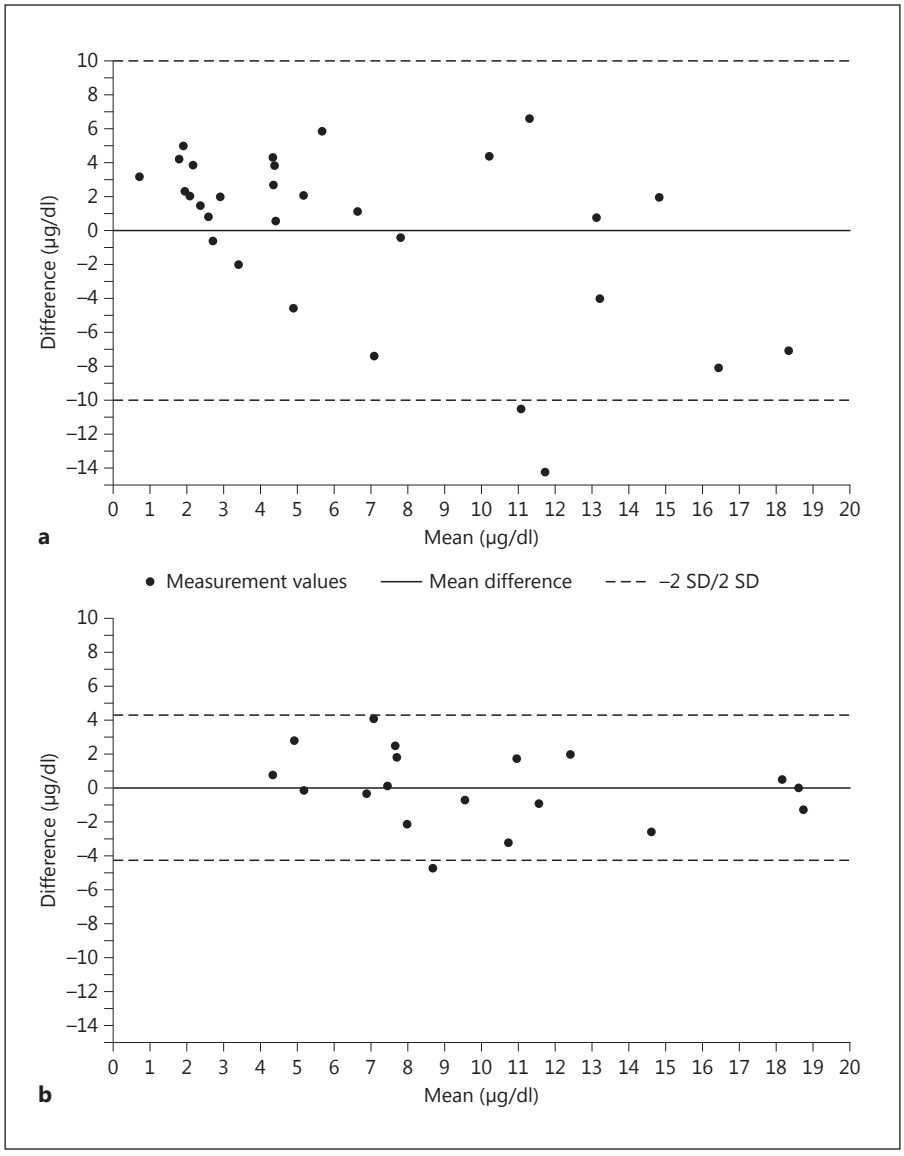

Fig. 1. Agreement between the two measurement methods of determining plasma cortisol (directly and via conversion from salivary cortisol data). **a** Preterm infants (n = 30). **b** Children (n = 19).

ies [14–19] in preterm infants and term neonates depicting the correlation of salivary and plasma cortisol (online suppl. fig. 4) could be included in the systematic review. These studies were published between 1987 and 2012, and the size of study cohorts ranged from 19 to 131 infants.

Patients

The identified studies investigated 350 neonates with regard to correlation of plasma and saliva cortisol levels including 183 preterm infants. In preterm infant cohorts, mean GA at birth ranged from 25.3 to 34.2 weeks and mean BW from 854 to 2,164 g (table 2). Three [15, 17, 19] of four [15–17, 19] identified studies on correlation between plasma and salivary cortisol in preterm infants did not report on exact weight and postmenstrual age of investigated infants at sample recovery. The rate of infants that could not be studied because of inadequate saliva sample size was only reported by Matsukura et al. [17]. In this study more than 50% of preterm infants (9/17) could not be investigated at 2 weeks of age because there was insufficient saliva recovered. Results of identified studies are summarized in table 2.

Methods of Saliva Collection

Saliva collection practice differed remarkably between studies: Francis et al. [14] collected saliva in term neo-

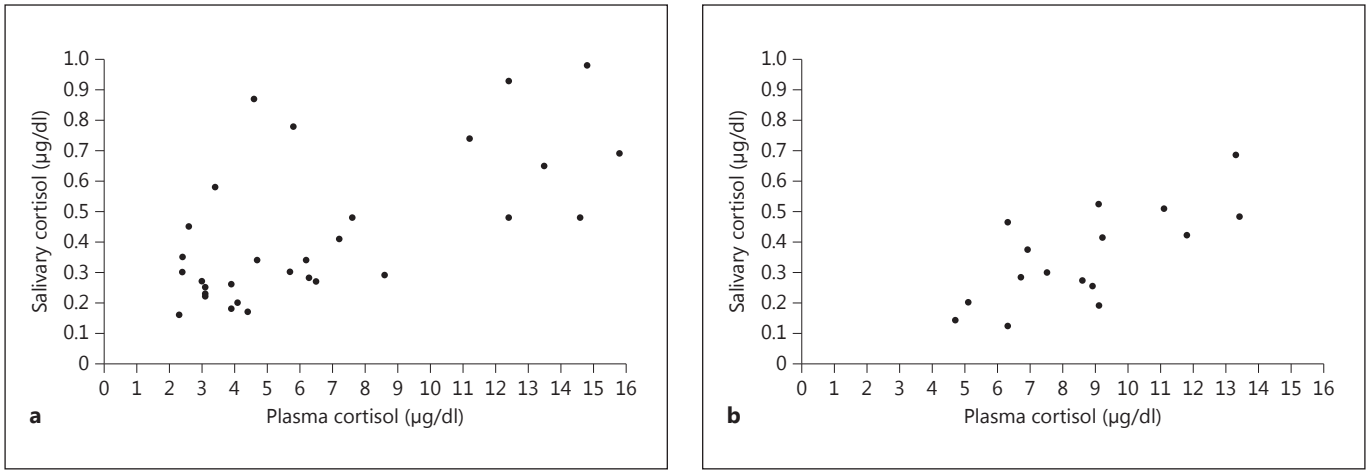

Fig. 2. Correlation between cortisol concentrations in plasma and saliva. **a** Correlation between cortisol concentrations in 30 matched samples of plasma (x) and saliva (y) obtained from 30 preterm infants (Spearman r = 0.6, 95% CI 0.31–0.79). **b** Correlation between cortisol concentrations in 19 matched samples of plasma (x) and saliva (y) obtained from 19 children/young adults (Spearman r = 0.8, 95% CI 0.53–0.92).

Table 2. Characteristics and results of identified studies on correlation between plasma and salivary cortisol levels in preterm infants and term neonates

	Francis et al. [14]	Kurihara et al. [18]	Calixto et al. [15]	Chou et al. [16]	Matsukura et al. [17]	Ng et al. [19]	Own data
Study characteristics							
Publication year	1987	1996	2002	2011	2012	2012	2014
Number of infants	36	131	48	51	19	65	30
Number of analyzed paired samples	41	72	144	51	52	NR	30
Mean gestational age at birth, weeks	term	39.5	30.6	34.2	26.7	25.3	30.7
Mean birth weight, g	NR	3,111	1,085	2,164	854	NR	1,372
Assay for salivary cortisol	RIA	RIA	RIA	RIA	EIA	EIA	EIA
Results							
Correlation coefficient (r)	0.83	0.718; $p < 0.0001$	0.67; $p < 0.0001$	0.481; $p = 0.0003$	0.78; $p < 0.0001$	0.44; $p = 0.001$	0.6; $p = 0.0001$
95% CI for correlation coefficient	NR	NR	NR	NR	0.64–0.87	NR	0.31–0.79
Sensitivity/specificity of salivary cortisol for detecting adrenal insufficiency	NR	NR	NR	NR	NR	NR	0.66/0.62

NR = Not reported; RIA = radioimmunoassay; EIA = enzyme immunoassay.

nates by an aspiration procedure using a suction catheter and facilitated salivation by citric acid. They obtained a saliva volume >25 µl in 94% of samples. Calixto et al. [15] also stimulated salivation by citric acid and reported the collection of 500 µl saliva. In the study by Kurihara et al. [18], unsweetened lemon crystals were given to obtain sufficient saliva if necessary. The three most recent studies did not perform procedures to stimulate salivation and collected saliva by using Salivette (Sarstedt), standard universal swabs or Sorbette sponges (Salimetrics).

Relationship of Salivary and Plasma Cortisol Levels

All identified studies reported exclusively correlation coefficients. These ranged from 0.44 to 0.83 with p values ranging from $p < 0.0001$ to $p = 0.0003$. A 95% CI for the correlation coefficient was only given by Matsukura et al. [17] (r = 0.78 (95% CI 0.64–0.87). None of the studies reported sensitivity and specificity of salivary cortisol to detect adrenal insufficiency.

Discussion

We found no satisfying agreement between cortisol levels derived from salivary cortisol determinations compared to those found through direct measurements in plasma. Furthermore, sensitivity and specificity of salivary cortisol in detecting adrenal insufficiency was low. These findings show that salivary cortisol is no helpful diagnostic tool in the ascertainment of adrenal insufficiency in preterm infants. Our study is the first to report sensitivity and specificity of salivary cortisol to detect adrenal insufficiency in preterm infants in the published medical literature.

To our best knowledge this is the first study reporting results of plasma and salivary cortisol determinations in preterm infants and older children using identical analytical and appropriate statistical methods for a reliable comparison of plasma and salivary cortisol in these two age cohorts. Another strength is the exact illustration of the relationship between plasma and salivary cortisol values in the lower range of plasma cortisol levels.

In many of the preterm infants enrolled, we could not analyze salivary cortisol because of insufficient quantity of collected saliva. This is a limitation of our method of saliva collection. Here we are in line with Matsukura et al. [17] who reported a comparable rate of preterm infants in whom sampling of adequate saliva volume failed in the early postnatal period. On account of possible interference effects on salivary cortisol [20], we refrained from stimulating saliva production by citric acid. Aspiration procedures were also avoided for the same reason. Collecting adequate amounts of saliva for laboratory measurements without stimulation of saliva production seems to be a difficult task in preterm infants and improvement of collection methods should be addressed in future studies. Furthermore, it seems advisable to adopt radioimmunoassay methods requiring less sample volume [21] if saliva cortisol determination is to be applied in premature infants.

For substituting plasma cortisol by salivary cortisol levels a satisfying agreement between the two measurement methods must be proven. Data demonstrating correlation coefficients with low p values are no adequate basis for this purpose and further statistical methods, such as the method proposed by Bland and Altman [12] should be applied. The latter showed unsatisfactory agreement for our data between plasma cortisol levels obtained via conversion of saliva cortisol data and directly measured plasma levels (fig. 2). Thus, salivary cortisol measurements are not suitable for replacing plasma cortisol determinations in preterm infants. In line with the insufficient agreement is the low sensitivity and specificity of salivary cortisol in the detection of adrenal insufficiency.

Furthermore, the importance of values in the lower range of plasma cortisol – which is of utmost interest for diagnosis of adrenal insufficiency – is weakened by unexpectedly high salivary cortisol levels in some preterm infants (see fig. 2a). The observed remarkable variation of salivary cortisol levels in the lower range of plasma cortisol may be attributed partially to potential cortisol contamination of the oral cavity due to frequent milk feeding in preemies [22]. Furthermore, a diminished concentration of cortisol-binding globulin (CBG) in very preterm infants may contribute to the above-mentioned phenomenon: Francis et al. [14] hypothesized a disproportionate rise in salivary cortisol compared with plasma total cortisol when CBG becomes saturated with cortisol because salivary cortisol represents free plasma cortisol. Yet interactions between albumin and CBG plasma concentrations in preterm infants and salivary cortisol are not investigated sufficiently.

Potential determination of urinary adrenal steroids might be a promising alternative in search of non-invasive diagnostic methods for diagnosing adrenal insufficiency in preterm infants [23, 24]. However, low urinary output, frequently associated with adrenal insufficiency, is hampering this approach.

Systematic review of the literature revealed a considerable diversity between studies in saliva sampling procedures. The resulting correlation coefficients between plasma and saliva cortisol levels in preterm infants also show a wide range (see table 2), and are not informative in terms of potential substitution of plasma cortisol by salivary cortisol measurements as agreement between methods was not demonstrated. Additionally, several investigators [14, 15, 17] calculated correlation coefficients using several samples per infant (see table 2), thus usually minimizing variance. The retrieved heterogeneity for cortisol plasma-saliva correlation coefficients in preterm infants again reinforces the dubiety of salivary cortisol for the diagnostic work-up of adrenal insufficiency in preterm infants.

Conclusions

Substitution of plasma cortisol by salivary cortisol determination cannot be recommended in preterm infants because of an unsatisfactory agreement between methods.

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